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Transport of Metal-binding Peptides by HMT1, A Fission Yeast ABC-type Vacuolar Membrane Protein*

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The *Schizosaccharomyces pombe hmt1* gene encodes an ABC (ATP-binding cassette)-type protein essential for Cd^{2+} tolerance. Immunoblot analysis of subcellular fractions indicates that the native HMT1 polypeptide is associated with the vacuolar membrane. Vacuolar membrane vesicles were purified from strains that hyperproduce, or are deficient in, the HMT1 protein. *In vitro* transport of radiolabeled substrates by these vesicles indicates that HMT1 is an ATP-dependent transporter of phytochelatins, the metal-chelating peptides involved in heavy metal tolerance of plants and certain fungi. Vacuolar vesicles containing HMT1 are capable of taking up both apo-phytochelatins and phytochelatin-Cd^{2+} complexes. HMT1 activity is sensitive to antibodies directed against this protein and to vanadate, but not to inhibitors affecting the vacuolar proton ATPase or ionophores that abolish the pH gradient across the vacuolar membrane. Vacuolar uptake of Cd^{2+} and of a glutathione conjugate were also observed, but are not attributable to HMT1. These studies highlight the importance of the yeast vacuole in detoxification of xenobiotics.

ABC[1] (ATP-binding cassette)-type proteins represent one of the largest known families of membrane transporters (1). Members of this superfamily are characterized by the presence of a highly conserved nucleotide-binding domain that is associated with a more variable region capable of spanning the membrane multiple times. DNA sequence analysis indicated that the *Schizosaccharomyces pombe hmt1* (*h*eavy *m*etal *t*olerance *1*) gene, essential for Cd^{2+} tolerance, encodes an ABC-type protein (2). Most eukaryotic ABC-type polypeptides exhibit a duplicated structure, containing two transmembrane and two nucleotide-binding domains. HMT1, on the other hand, has only one of each. The few known eukaryotic ABC-type proteins that exhibit this nonduplicated arrangement reside in internal membranes: rat PMP70 (3) and its human homologue (4) in the peroxisome and TAP1 and TAP2 in endoplasmic reticulum and cis-Golgi (5). Previously we reported that an HMT1-β-galactosidase fusion protein was localized to the fission yeast vacuole (2).

ABC-type proteins can mediate tolerance to a wide diversity of cytotoxic agents. Multiple drug resistance proteins from mammals (reviewed in Ref. 6) and yeast (7, 8) decrease the toxicity of a variety of anti-tumorigenic drugs. *Leishmania* IgptA confers tolerance to methotrexate and arsenite (9), and a number of bacterial transporters are involved in resistance to antibiotics and environmental toxins (reviewed in Ref. 10). Resistance is achieved by export of the toxic substances from the cell. The intracellular location of the HMT1-β-galactosidase chimera, on the other hand, suggested that this protein might represent the first example of an ABC-type transporter mediating resistance to a toxin by sequestration in an intracellular membrane-bound compartment. The nature of the substrate transported by HMT1, and its role in heavy metal tolerance of fission yeast were not clear.

Plants and certain fungi, including *S. pombe*, respond to heavy metals by inducing synthesis of small peptides known as phytochelatins (PCs) (11–13). Unlike metallothioneins, PCs are not produced by mRNA translation but are enzymatically synthesized from glutathione (GSH, γ-Glu-Cys-Gly). PCs have the general structure $(\gamma\text{-Glu-Cys})_n\text{Gly}$, where $n = 2$–11, and like metallothioneins, chelate heavy metals by formation of thiolate bonds. Yeast and plant cells exposed to Cd^{2+} accumulate a low molecular weight (LMW) PC·Cd complex, consisting mostly of PCs and Cd^{2+}, and a high molecular weight (HMW) PC·Cd·S^{2-} complex, containing acid labile sulfide (14–16). Genetic and biochemical analyses suggest that production of the sulfide moiety in the HMW PC·Cd·S^{2-} complex involves the purine biosynthetic pathway (17, 18). The HMW complex, a CdS crystallite coated by PC peptides (19), has a higher Cd^{2+}-binding capacity than LMW PC·Cd, and the Cd^{2+} ions are less susceptible to acid displacement (20). Although detailed structures for HMW and LMW PC·Cd complexes are not available, it is clear that both are heterogeneous oligomeric aggregates containing multiple PC peptides of variable length.

Since overexpression of *hmt1* in yeast cells confers enhanced Cd^{2+} tolerance, as well as accumulation of the metal (2), it was postulated that HMT1 was involved in vacuolar sequestration of heavy metals or metal-peptide complexes. In this report we describe the localization of native HMT1 protein to vacuolar membranes and the discovery of an ATP-dependent PC transport activity in vacuolar membrane vesicles. We present evidence showing that HMT1 is responsible for this activity and describe the substrate specificity and energy requirements of this transporter. In addition, we observed Cd^{2+} and glutathione conjugate transport activities in fission yeast vacuolar vesicles lacking detectable amounts of HMT1 protein.

MATERIALS AND METHODS

Yeast Strains—The *S. pombe* strain Sp223 (h^-, *ade6–216, leu1–32, ura4–294*) is used as a wild type control in these experiments. The

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[1] The abbreviations used are: ABC, ATP-binding cassette; AMP-PNP, adenosine 5′-(β,γ-imido)triphosphate; GSH, glutathione; GSDNP, dinitrophenyl-*S*-glutathione; HMW, high molecular weight; LMW, low molecular weight; PC, phytochelatin; hmt, heavy metal tolerance; MES, 4-morpholineethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid; HPLC, high performance liquid chromatography.

hmt1$^-$ mutant, LK100, was derived from Sp223 by ethyl methanesulfonate-induced mutagenesis (2). In most analyses LK100 contains either the yeast expression vector pART1 or pDH35 (pART1 containing an *hmt1* cDNA) (2). Cultures were grown at 30 °C in S.D. (6.7 g of Difco yeast nitrogen base, 20 g of glucose/liter, adenine and uracil at 20 μg/ml).

Antibodies Directed against HMT1—A 760-base pair *Cla*I-*Mae*III cDNA fragment encoding amino acids 553–806 of HMT1 was subcloned into the Qiagen pQIA16/17 vector system (pDH60). A 29-kDa His-tagged product, purified by Ni-agarose affinity chromatography from *Escherichia coli*, was used to immunize rabbits 622 and 623. Antisera 622 and 623 recognized a 29-kDa peptide in *E. coli*/pDH60 that was isopropyl β-thiogalactopyranoside inducible, but was not detected in *E. coli*/pQIA16. Preimmune sera failed to recognize this protein. IgG was purified from 622 and 623 antisera using Protein A-agarose (Bio-Rad).

Plasmid pDH30 (2), containing an *hmt1* cDNA in the pBluescript SK vector (Stratagene), was used for *in vitro* transcription/translation by the TnT T7 system (Promega) in the presence of [^{35}S]Met (1141 Ci/mmol, DuPont). Canine pancreatic microsomal membranes (Promega) were added according to the manufacturer's instructions. Translation products were immunoprecipitated using Protein A-agarose or directly separated by 10% SDS-polyacrylamide gel electrophoresis for fluorography.

Isolation of Vacuole Membrane Vesicles—*S. pombe* vacuolar vesicles were isolated as described previously (2) with the following modifications. All buffers contain 2 mM benzamidine, 4 μg/ml leupeptin, 4 μg/ml pepstatin A, and 1 mM phenylmethylsulfonyl fluoride. Spheroplasts were lysed in Buffer A (1.6 M sorbitol, 10 mM MES-Tris, pH 6.9, 0.5 mM $MgCl_2$) with a glass homogenizer. Unlysed cells were pelleted by centrifugation at 2,500 × *g* for 10 min. A half-volume of Buffer B (Percoll containing 1.6 M sorbitol, 10 mM MES-Tris, pH 6.9, 1 mM $MgCl_2$) was added to the supernatant (final Percoll concentration of 30%) and the vacuoles pelleted at 15,000 × *g* for 30 min. The vacuole pellet was resuspended in Buffer A with a homogenizer, mixed with a half-volume of Buffer B, layered on a cushion of equal volumes of Buffers A and B (50% Percoll), and pelleted as before. The vacuolar pellet was resuspended in Buffer C (100 mM KCl, 10 mM MES-Tris, pH 6.9, 5 mM $MgCl_2$) and vacuolar membrane vesicles pelleted at 7,000 × *g* for 10 min. Vacuolar vesicles were resuspended in 0.5–1.5 ml of Buffer C containing 0.25 M sucrose and frozen in liquid nitrogen as 50-μl aliquots.

Enzyme Assays—Specific activities of marker enzymes were compared in total membranes and vacuolar membrane vesicles. To prepare total membranes for marker enzyme analysis, an aliquot of the 2,500 × *g* cell-free supernatant from the vacuolar preparation was diluted 10-fold with Buffer C and membranes pelleted by centrifugation at 100,000 × *g*. The particulate fraction was resuspended in Buffer C with 0.25 M sucrose and frozen in liquid nitrogen. α-Mannosidase, cytochrome *c*-reductase, dipeptidyl aminopeptidase B, glucose-6-phosphate dehydrogenase, and ATPase activities were measured as described (21). Succinate dehydrogenase (22) and Golgi GDPase (23) were determined as reported. Quenching of acridine orange fluorescence was measured with a Perkin Elmer LS5 Fluorescence spectrometer (24), except that EDTA was omitted from the reaction buffer. Protein concentration was determined using the Bradford assay (Bio-Rad) containing 0.05% CHAPS. The values obtained by modified Lowry assay on trichloroacetic acid-precipitated material were not significantly different from those generated with the Bradford assay.

Synthesis and Purification of Substrates—Sp223 cells grown in 800 ml of S.D. to an A_{600} of 0.6–0.8 were incubated with 0.25 mCi/liter of [^{35}S]Cys (Amersham, 1300 Ci/mmol) and 0.4 mM $CdSO_4$ for 8 h. Cells were lysed with glass beads in 1.5 volumes of 50 mM Tris, pH 7.6, 100 mM KCl, 0.1 mM $CdCl_2$, 0.5% isoamyl alcohol and centrifuged at 10,000 × *g* for 10 min. Elution of PC·Cd complexes from DEAE-Sephacel (Pharmacia Biotech Inc.) with a linear KCl gradient (0.2–0.5 M, 10 mM Tris, pH 7.6) generated two well resolved peaks of ^{35}S-labeled material. Extracts labeled with $^{109}CdCl_2$ instead of [^{35}S]Cys produced an identical column profile. ^{35}S-Labeled fractions were reduced in volume to 4 ml *in vacuo* and applied to a Sephadex G-50 column as described (2). The positions of ^{35}S peaks in the G-50 elution profile were consistent with purification of HMW and LMW PC·Cd complexes. These peaks could also be labeled by addition of a $^{109}CdCl_2$ tracer to the sample before loading. Concentrated G-50 column fractions, desalted by Sephadex G-10 chromatography, were used in transport studies. HPLC analysis of the PC·Cd complexes (essentially as described in Ref. 25, except that trifluoroacetic acid was substituted with 0.1% H_3PO_4) indicated that the PCs were mostly of n = 2–3, in agreement with Grill *et al.* (26), and did not contain GSH. Apo-PCs were prepared from the LMW [^{35}S]PC·Cd complex by acidification to pH 2.0 with 1 N HCl followed by HPLC separation (20). ^{35}S-Labeled HPLC fractions were neutralized and desalted by Sephadex G-10 chromatography. The LMW [^{35}S]PC·Cd complex reconstituted from apo-PCs and Cd^{2+} was indistinguishable from the native complex by Sephadex G-50 gel filtration or as a substrate in transport assays. A LMW [^{35}S]PC·Cd complex could also be derived from purified HMW [^{35}S]PC·Cd·S^{2-} complex by acidification with 1 N HCl to pH 2.0 in the presence of 10 mM β-mercaptoethanol (20). After out-gassing the evolved H_2S, the reaction was neutralized using NaOH, and subjected to Sephadex G-50 gel chromatography. Greater than 90% of the counts present in the HMW [^{35}S]PC·Cd·S^{2-} complex were recovered from G-50 as a peak indistinguishable from the native LMW [^{35}S]PC·Cd complex. This same peak could also be labeled by addition of a ^{109}Cd tracer. PC concentration was measured with Ellman's reagent (27) and interpolated on a GSH standard curve. Because native PC·Cd complexes are heterogeneous regarding number of peptides per complex and length of peptides, PC concentrations are reported as GSH equivalents. Unlabeled PC·Cd complexes were prepared in essentially the same manner. Cd^{2+} content of column fractions was quantified by atomic absorption spectroscopy as described previously (2). ^{109}Cd was incorporated into LMW PC·Cd complexes of known Cd^{2+} and thiol concentration by incubation with $^{109}CdCl_2$ for 30 min on ice. Free Cd^{2+} ions were separated from the LMW [^{109}Cd]PC·Cd complex by G-10 chromatography.

[^{3}H]GSSG was prepared from [^{3}H]GSH (300 mCi/mmol, DuPont) (28). The 2,4-dinitrophenyl-*S*-glutathione conjugate (GSDNP) was enzymatically synthesized from 1-chloro-2,4-dinitrobenzene (Sigma) and [^{3}H]GSH by glutathione *S*-transferase (Sigma) (29). [^{3}H]GSDNP was separated from its precursors by thin layer chromatography and quantified by absorbance at 340 nm.

Vesicle Filtration Assay—Vesicle filtration assays were performed at 30 °C in 10 mM MES-Tris, pH 7.8, 20 mM KCl, 5 mM $MgCl_2$, 10 mM creatine phosphate, and 100 μg/ml creatine kinase (Buffer D). 50 μl of thawed vacuolar vesicles (20–50 μg of protein) were resuspended in 200 μl of Buffer D, ATP added to a final concentration of 3 mM, and 50 μl of radiolabeled substrate added 30 s later. At the times indicated in the figures, 50-μl aliquots from the reaction were diluted to 1 ml with ice-cold Buffer D, filtered immediately under light vacuum through a 25-mm GF-C filter (Whatman), and washed with 5 ml of Buffer D. For Cd^{2+} transport, 50 μl of 48 μM $^{109}CdCl_2$, 100 mCi/mmol (DuPont) was used (8 μM final concentration), and the wash contained 2 mM $CdCl_2$ to reduce nonspecific binding of Cd^{2+} to membranes. For [^{3}H]GSDNP uptake studies, a final concentration of 50 μM GSDNP was used. For PC uptake studies, a solution containing 50,000 cpm of ^{35}S-labeled PCs (4–8 μM GSH equivalents) was used. For measurement of [^{109}Cd]PC·Cd uptake, the LMW [^{109}Cd]PC·Cd substrate was present at a final concentration of 8 μM Cd^{2+} and 16.4 μM GSH equivalents. For antibody inhibition of PC uptake, an aliquot of vacuolar vesicles was mixed with Buffer D and incubated on ice for 1 h with preimmune serum, no antibody, or 300 μg of IgG derived from either antiserum 622 or 623. ATP and PC substrates were added and the vesicle filtration assay conducted as above.

RESULTS

hmt1 Encodes a 90-kDa Vacuolar Membrane Protein—An HMT1-β-galactosidase fusion protein had previously been localized to the vacuolar membrane of *S. pombe* (2). However, reports indicating that hyperproduction of a membrane protein may result in mislocalization to the vacuole (30) and that the vacuolar membrane may represent the default sorting pathway for yeast membrane proteins (31) suggested that addition of the 105-kDa β-galactosidase peptide to the carboxyl terminus of HMT1 may have altered its subcellular sorting. Therefore, the intracellular location of native HMT1 remained uncertain.

A 29-kDa peptide, encompassing most of the HMT1 ATP-binding cassette domain, was synthesized in *E. coli*, purified, and used to raise antibodies (antisera 622 and 623). Both antisera recognized a 90-kDa protein in *S. pombe* (Fig. 1*A*) that was not detected by preimmune sera and is consistent with the 90.5-kDa molecular mass predicted for HMT1. The 90-kDa protein was associated with the P100 (100,000 × *g* precipitable) particulate fraction of *S. pombe* extracts (*lane g*), but is not detected in the S100 supernatant (*lane e*), as expected of an integral membrane protein.

Vacuoles were prepared from *S. pombe* spheroplasts as de-

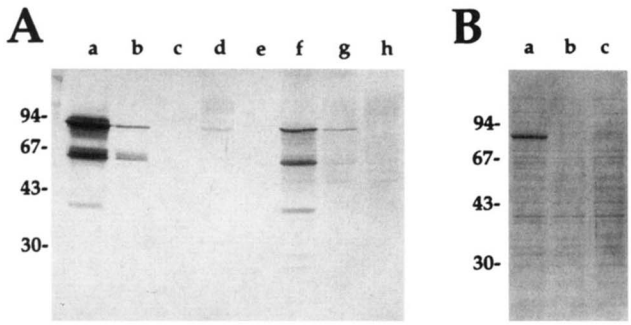

FIG. 1. *A*, immunoblot of *S. pombe* proteins using antiserum 622. Lanes contain 2 μg of protein from vacuolar membrane vesicles prepared from: (*a*) LK100/pDH35 (*hmt1*$^-$ mutant complemented by an *hmt1* cDNA in pART1); (*b*) Sp223/pART1 (*hmt1*$^+$ strain with empty vector); and (*c*) LK100/pART1. 40 μg of protein from: (*d*) a P100 (100,000 × *g* precipitable) pellet derived from the supernatant overlying the purified vacuole pellet and (*e*) the S100 (supernatant) fraction of Sp223/pART1. 50 μg of protein from the P100 fractions of extracts made from: (*f*) LK100/pDH35; (*g*) Sp223/pART1; and (*h*) LK100/pART1. The blot was overdeveloped to show the lack of HMT1 in LK100/pART1 lanes. The smaller bands (62 and 39 kDa) recognized by the antibody varied in intensity in different experiments and are probably produced by proteolytic degradation of HMT1. Similar effects have been reported with preparations of mammalian P-glycoprotein (32). *B*, Coomassie stained replica of the portion of the gel (*lanes a-c*) shown in *A* that contains vacuolar membranes.

TABLE I

Comparison of specific activities of marker enzymes in vacuolar membrane vesicles and total cellular membranes

Total cellular membranes and vacuolar membrane vesicles were prepared as described under "Materials and Methods." Specific activities are reported as nmol/min/mg protein. The marker enzymes represent the following cellular compartments: cytochrome *c*-reductase for endoplasmic reticulum, succinate dehydrogenase for mitochondria, glucose-6-phosphate dehydrogenase for cytoplasm, GDPase for Golgi membrane, α-mannosidase and dipeptidyl aminopeptidase B for vacuoles. Mitochondrial, vacuolar, and plasma membrane ATPase activities represent ATPase activity sensitive to 2 mM azide, 1 μM bafilomycin, and 100 μM vanadate, respectively.

Marker enzyme	Specific activity		Ratio
	Total	Vacuole	
			vacuole/total
Cytochrome *c*-reductase	18.3	0.5	0.03
Succinate dehydrogenase	198	2.6	0.01
Glucose-6-phosphate dehydrogenase	390	4.0	0.01
GDPase	0.095	<0.001	<0.01
α-Mannosidase	9.3	300	32.0
Dipeptidyl aminopeptidase B	110	3,800	34.5
Mitochondrial ATPase	10,700	<100	<0.01
Plasma membrane ATPase	1,910	<100	<0.05
Vacuolar ATPase	3,050	74,500	24.4

scribed under "Materials and Methods." Compared to the particulate fraction of a total cellular extract, purified vacuolar vesicles are highly enriched in vacuolar marker enzymes and impoverished in markers for mitochondrial, Golgi, endoplasmic reticulum, and plasma membranes (Table I). In the immunoblot in Fig. 1*A*, lanes loaded with vacuolar membranes (Fig. 1*A*, *lanes a* and *b*) exhibited enrichment of the *hmt1* product when compared with total cellular membranes (*lanes f* and *g*) despite containing 25-fold less protein. The supernatant overlying the vacuolar pellet was impoverished in HMT1 (*lane d*), suggesting that this protein is sorted primarily to the vacuolar membrane. Cd^{2+} treatment of wild type cultures did not alter levels of the 90-kDa protein (not shown).

The 90-kDa polypeptide was not detected in total (*lane h*), or vacuolar (*lane c*), membranes derived from the Cd^{2+}-sensitive LK100 strain, which bears an *hmt1*$^-$ allele with a nonsense mutation in the 5′ region of the gene (2). Strains transformed with the pDH35 plasmid accumulate high levels of the *hmt1* mRNA (2) and 90-kDa peptide (Fig. 1*A*, *lanes a* and *f* and *B*, *lane a*).

In Vitro Transcription/Translation of hmt1 cDNA—*In vitro* transcription/translation of the *hmt1* cDNA generated a 90-kDa product, but only if canine pancreatic microsomes or Triton X-100 detergent were present in the reaction (Fig. 2*A*). *In vitro* translation of other integral membrane proteins (33, 34) also depend on these additives. The apparent M_r of *hmt1* translation products obtained with Triton X-100 and canine microsomes was the same, suggesting that the *hmt1* gene product was not glycosylated *in vitro* upon insertion into microsomes. The *in vitro* translation product also co-migrated with the 90-kDa band present in vacuolar membrane extracts (see Fig. 1*B*), suggesting that HMT1 may not be glycosylated *in vivo* either. Translation products were immunoprecipitated with antisera 622 and 623 but not preimmune sera (Fig. 2*B*). In addition to the 90-kDa product, several smaller peptides generated in the transcription/translation reaction were also recognized by the antisera. Since the antibodies are directed to the carboxyl-terminal ABC domain of HMT1, the smaller products could derive from use of cryptic transcription and/or translation start signals present in the *hmt1* cDNA. Highly hydrophobic proteins are translated far less efficiently than soluble

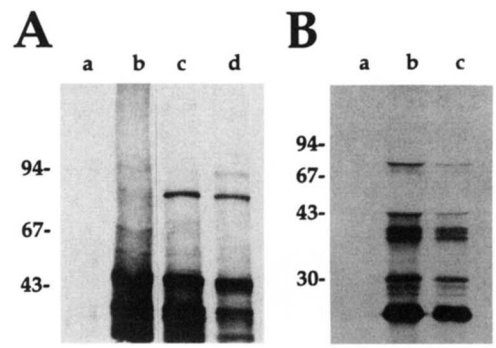

FIG. 2. ***In vitro* transcription/translation of *hmt1* cDNA.** *A*, fluorograph of an SDS-polyacrylamide gel electrophoresis gel containing [^{35}S]Met labeled *in vitro* translation products derived from: (*a*) pBluescript SK+ canine pancreatic microsomes; (*b*) pDH30 (pBluescript SK containing an *hmt1* cDNA) without additives; (*c*) pDH30 + 1% Triton X-100; (*d*) pDH30 + canine pancreatic microsomes. *B*, fluorograph of immunoprecipitated products from *in vitro* transcription/translation of pDH30 in the presence of 1% Triton X-100: (*a*) preimmune serum of rabbit 622; (*b*) anti-HMT1 antiserum 622; (*c*) anti-HMT1 antiserum 623.

polypeptides (up to 40-fold lower than the luciferase and *S. pombe ade2* gene products). Use of the next downstream ATG codon, encoding Met-318, as a translational start site would generate a truncated peptide lacking the most hydrophobic region of HMT1. The M_r of seven of the eight smaller peptides present in the translation reaction mixture match the sizes predicted if downstream Met codons were used as intragenic translational start sites. Production of these smaller peptides is not dependent on microsomes or detergent; therefore, their translation might be more efficient than that of the full-length HMT1.

[^{35}S]PC Uptake Is HMT1-dependent—Possible functions of HMT1 include transport of heavy metals and/or PC-metal complexes. Vacuolar membrane vesicles isolated from mutant (LK100/pART) and HMT1 hyperproducing (LK100/pDH35) strains were tested for uptake of radioactively labeled substrates by the standard vesicle filtration assay. Vesicles derived from the HMT1 hyperproducer exhibited ATP-dependent uptake of *in vivo* labeled LMW [^{35}S]PC·Cd complex (Fig. 3*A*). No activity was

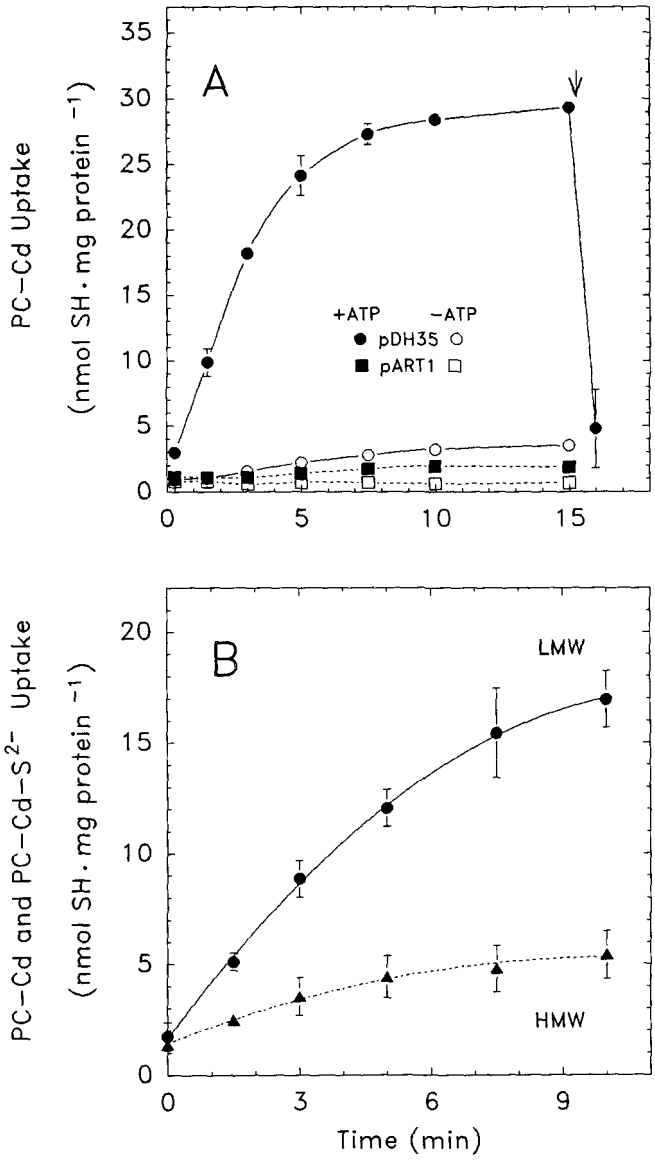

FIG. 3. **Uptake of ^{35}S-labeled PC·Cd complexes by purified vacuolar membrane vesicles.** *A*, ATP-dependent uptake of LMW PC·Cd. Vacuolar vesicles (20–40 μg of protein) prepared from the *hmt1*$^-$ mutant LK100 bearing the pART1 vector containing an *hmt1* cDNA (pDH35) (●, ○) or no insert (■, □) were incubated with 8 μM GSH equivalents of LMW [^{35}S]PC·Cd complex (50,000 cpm) in the presence (●, ■) or absence (○, □) of 3 mM ATP and an ATP regeneration system. Aliquots were taken at the times indicated and substrate uptake determined by the vesicle filtration assay. *Arrow* indicates addition of Triton X-100 to 0.02%. *B*, comparison of uptake of LMW PC·Cd and HMW PC·Cd·S^{2-} complex by vacuolar vesicles. Vesicles from LK100/pDH35 were incubated with an equal number of counts (50,000 cpm representing 6 μM GSH equivalents) of LMW [^{35}S]PC·Cd (●) or HMW [^{35}S]PC·Cd·S^{2-} (▲) in the presence of ATP. *Bars* represent S.E.

observed at 0 °C, in the absence of ribonucleotides, or with AMP, ADP, or the non-hydrolyzable ATP analog AMP-PNP. Inclusion of a creatine kinase/creatine phosphate ATP-regeneration system resulted in a 1.6-fold increase in initial uptake velocity and maintained linearity of uptake for a longer period of time. LMW PC·Cd uptake has a K_m for ATP of 0.38 ± 0.01 mM (Fig. 4). 3 mM UTP, CTP, or GTP sustained 97 ± 3, 62 ± 1, or 54 ± 1%, respectively, of the initial velocity measured with 3 mM ATP in the absence of a regeneration system. Addition of Triton X-100 to vesicles loaded with radiolabeled PCs resulted in a decrease of ^{35}S counts retained on the filter. Furthermore, a decrease in the initial velocity of [^{35}S]PC uptake was observed upon reduction of

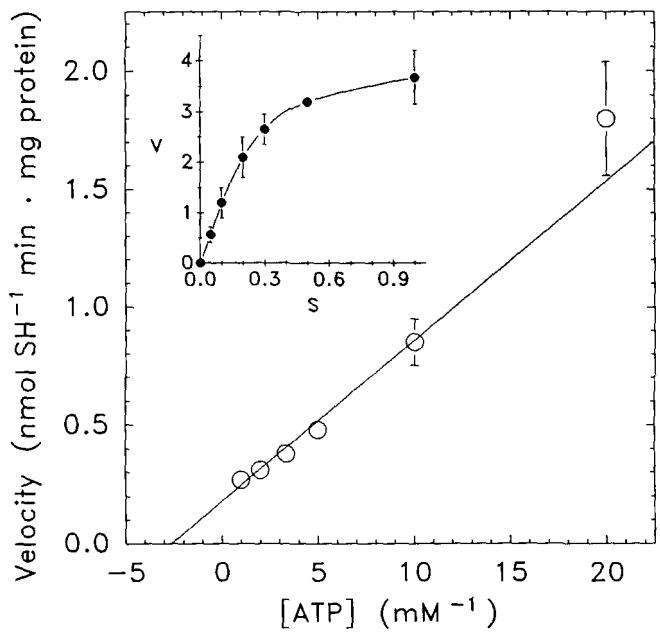

FIG. 4. **Dependence of PC·Cd uptake on ATP concentration.** Double reciprocal plot of data used to calculate the K_m of HMT1 for ATP. Linear regression curve was calculated as described (35). *Inset*: initial velocity of LMW [^{35}S]PC·Cd complex uptake (calculated as in Table II) by LK100/pDH35 vacuolar membrane vesicles measured in the presence of an ATP regeneration system and varying concentrations of ATP. *Bars* represent S.E.

the internal vesicle space (Fig. 5), suggesting that PCs were transported into the vacuolar vesicles rather than binding to the outside of the membrane.

Unlike vesicles derived from the HMT1 hyperproducing strain, LK100/pART1 vacuolar membrane vesicles exhibited no ATP-dependent transport of the LMW [^{35}S]PC·Cd complex (Fig. 3*A*). Very little activity was observed with membrane vesicles derived from Sp223/pART1 (not shown), probably because of the relatively low level of HMT1 protein present in wild type vacuoles (compare *lanes a* and *b* in Fig. 1*B*) and low specific activity of the *in vivo* labeled PC substrate. The differences in PC uptake activity among the various strains was not due to differential labilities of vacuolar vesicles. Transport competency of the isolated vacuolar vesicles were similar with regards to Cd^{2+} and GSDNP (see below). Additionally, vacuolar vesicles from all three strains exhibited comparable levels of ATP-dependent [^{14}C]arginine uptake (not shown), an activity that is also present in vacuoles of *Saccharomyces cerevisiae* (36) and *Neurospora crassa* (37).

One of the polyclonal antibodies directed against HMT1 inhibited the PC transport activity *in vitro*. Incubation of vacuolar vesicles with 1 mg/ml IgG purified from antiserum 622 resulted in a 63% reduction in the initial velocity of PC uptake (determined as in Table II) from 2.8 ± 0.6 to 1.3 ± 0.1 nmol/min/mg. Increasing antibody concentration did not further inhibit PC uptake. IgG purified from antiserum 623 had no effect on PC transport even at concentrations above 2 mg/ml IgG. The lack of PC uptake by vacuoles derived from the HMT1-deficient mutant, the high level of ATP-dependent PC transport observed in vacuolar vesicles prepared from the HMT1 hyperproducing strain, and the inhibition of PC uptake by antibodies directed against HMT1, all indicate that the *hmt1* gene encodes the PC transporter.

Vacuolar vesicle uptake of labeled PCs was challenged with a series of inhibitors (Table II). Vanadate, which inhibits the activity of a number of ABC-type proteins, significantly inhibited PC uptake with an IC_{50} of 0.082 ± 0.004 mM. Bafilomycin

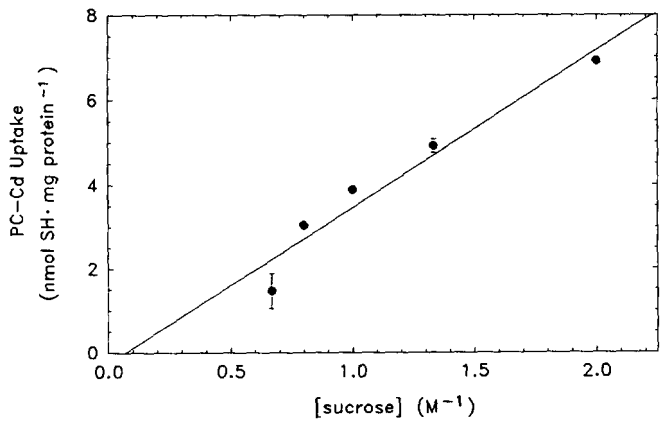

FIG. 5. **Inhibition of PC·Cd uptake by reduction of internal vesicle space.** Uptake of LMW [^{35}S]PC·Cd complex was measured as described in the legend to Fig. 3 in the presence of 0.5, 0.75, 1.0, 1.25, and 1.5 M sucrose. The initial uptake velocity (determined as in Table II) is plotted against the reciprocal of the sucrose concentration. *Bars* represent S.E.

TABLE II
Effect of inhibitors on uptake of Cd^{2+} or LMW PC·Cd by vacuolar vesicles

Uptake was measured in LK100/pDH35 vesicles. Initial uptake velocities were determined as the slope of the transport curve during the first 3 min of the reaction (containing at least three time points), and are expressed relative to value obtained in the absence of the inhibitor. All values represent ATP dependent uptake, *i.e.* the difference between substrate retained on the filters in the presence and absence of ATP at any given time point. Reactions included an inhibitor, 3 mM ATP, an ATP regeneration system, and either 8 μM Cd^{2+} or 8 μM GSH equivalents of the LMW PC·Cd complex.

Inhibitor	Concentration	Substrate uptake	
		LMW PC·Cd	Cd^{2+}
	mM	%	
No inhibitor		100	100
Nigericin	0.01	92 ± 6	<1[a]
Bafilomycin	0.001	105 ± 1	<1[a]
Valinomycin	0.01	103 ± 8	102 ± 5
Na_3VO_4	0.1	39 ± 3	95 ± 6
	0.05	70 ± 2	ND[b]
	0.01	88 ± 2	ND

[a] Initial velocity of Cd uptake in the presence of these inhibitors was usually equal or slightly less than the binding observed in the absence of ATP.
[b] ND, indicates value not determined.

A, an inhibitor of vacuolar ATPase, nigericin, a proton-K^+ ionophore, and valinomycin, a K^+ ionophore, had no significant effect, indicating that PC transport is not dependent on the ΔpH across the vacuolar membrane.

The HMW PC·Cd·S^{2-} Complex Is Not an Efficient Substrate—Cd-peptide complexes containing PCs of the same length and of the same specific activity were prepared by converting the ^{35}S-labeled HMW PC·Cd·S^{2-} complex to the LMW form through acid depletion of sulfide (20). Conversion was confirmed by a shift in the apparent M_r of the ^{35}S-labeled PC·Cd complex and a reduction in absorbance at 280 nm. Greater than 90% of the counts present in the HMW [^{35}S]PC·Cd·S^{2-} starting material were recovered from a Sephadex G-50 column as LMW [^{35}S]PC·Cd complex.

Initial velocity for ATP-dependent uptake of the LMW PC·Cd complex was 2.4 nmol/min/mg *versus* 0.7 nmol/min/mg for the HMW PC·Cd·S^{2-} complex (Fig. 3*B*). Estimating the precise difference in the rate with which these two complexes were taken up is confounded by the variable number of PC molecules per complex, over 25 in the HMW PC·Cd·S^{2-} complex (19) compared with 3–8 in the LMW PC·Cd complex (38). An additional consideration is that the HMW PC·Cd·S^{2-} complex can spontaneously break down to form the LMW PC·Cd form, thus it is unclear what proportion of counts taken up by the vacuolar vesicles represents bona fide transport of the HMW complex. Both of these factors lead to an overestimation of the rate of HMW PC·Cd·S^{2-} transport, and an underestimation of the difference in the relative affinity of HMT1 for these two substrates.

Apo-PCs as a Transport Substrate—To test if PCs need to be complexed with Cd^{2+} in order to be taken up by HMT1, Cd^{2+} was stripped from LMW [^{35}S]PC·Cd by acidification, and HPLC purified apo-[^{35}S]PCs were tested for uptake by vacuolar membrane vesicles. No significant difference was detected between uptake of apo-PCs and LMW PC·Cd and no enhancement of transport activity was observed upon supplementation of apo-PCs with $CdCl_2$ (not shown). This suggests that the apo-PC peptides can be transported by HMT1. However, Mg^{2+} has been provided at a relatively high concentration (5 mM) as transport of LMW PC·Cd is severely reduced in its absence. Although PCs have a much lower affinity for Mg^{2+} than metals ions such as Cd^{2+} or Cu^{2+} (39), it is possible that in the absence of Cd^{2+} the thousand-fold excess of Mg^{2+} drives the formation of a PC·Mg complex, which is taken up by vacuolar vesicles. Alternatively, trace amounts of other metals in the vacuolar vesicle preparation may be forming a complex with the apo-PCs.

Vacuoles Contain a Cd^{2+} Transporter That Is Not HMT1—Cells overexpressing *hmt1* hyperaccumulate Cd^{2+} (2). While this could be due to enhanced vacuolar sequestration of PC·Cd complexes it was also possible that HMT1 is directly involved in Cd^{2+} transport. Fig. 6*A* shows that ^{109}Cd was taken up by vacuolar membrane vesicles in an ATP-dependent manner. AMP, ADP, and non-hydrolyzable ATP analogs such as AMP-PNP did not support transport, and uptake was abolished at 0 °C. However, vesicles derived from LK100/pART1, LK100/pDH35 (Fig. 6*A*), or Sp223/pART (not shown) displayed very little difference in Cd^{2+} uptake, suggesting that HMT1 is not responsible for this activity. $^{109}Cd^{2+}$ uptake was unaffected by vanadate or valinomycin, but abolished by bafilomycin and nigericin (Table II), suggesting that Cd^{2+} transport is due to a proton antiporter dependent on the pH gradient generated by the vacuolar ATPase. Vacuolar vesicles will quench acridine orange fluorescence upon addition of ATP, although not if 0.5 μM bafilomycin is present, indicating that acidification of the intravesicular space is mediated by the vacuolar proton ATPase. Fluorescence is restored upon addition of 6 μM $CdCl_2$ to these vesicles, suggesting that Cd^{2+} influx is accompanied by proton efflux (not shown).

HMT1-dependent Uptake of Cd^{2+} Complexed with PCs—Because apo-PCs and Cd^{2+} ions can enter the vacuole through separate routes it is important to determine if PCs and Cd^{2+} are taken up as a complex as well. Inhibition of the V-ATPase with bafilomycin abolishes vacuolar vesicle uptake of ^{109}Cd but does not affect transport of ^{35}S-labeled PCs (Tables II and III). When vacuolar vesicles derived from the HMT1 hyperproducing strain LK100/pDH35 were incubated with a LMW [^{109}Cd]PC·Cd substrate in the presence of bafilomycin, ATP-dependent uptake of the ^{109}Cd radiolabel was observed (Table III). Vacuolar vesicles derived from HMT1-deficient LK100/pART, on the other hand, exhibit relatively little uptake of the LMW [^{109}Cd]PC·Cd complex in the presence of bafilomycin. As previously shown, vanadate impairs HMT1 transport of the LMW [^{35}S]PC·Cd complex but does not inhibit the Cd^{2+}/H^+ antiporter (Table II). Uptake of LMW [^{109}Cd]PC·Cd by LK100/pDH35 vesicles was reduced by 69% in the presence of vanadate (Table III), similar to the 61% inhibition observed for

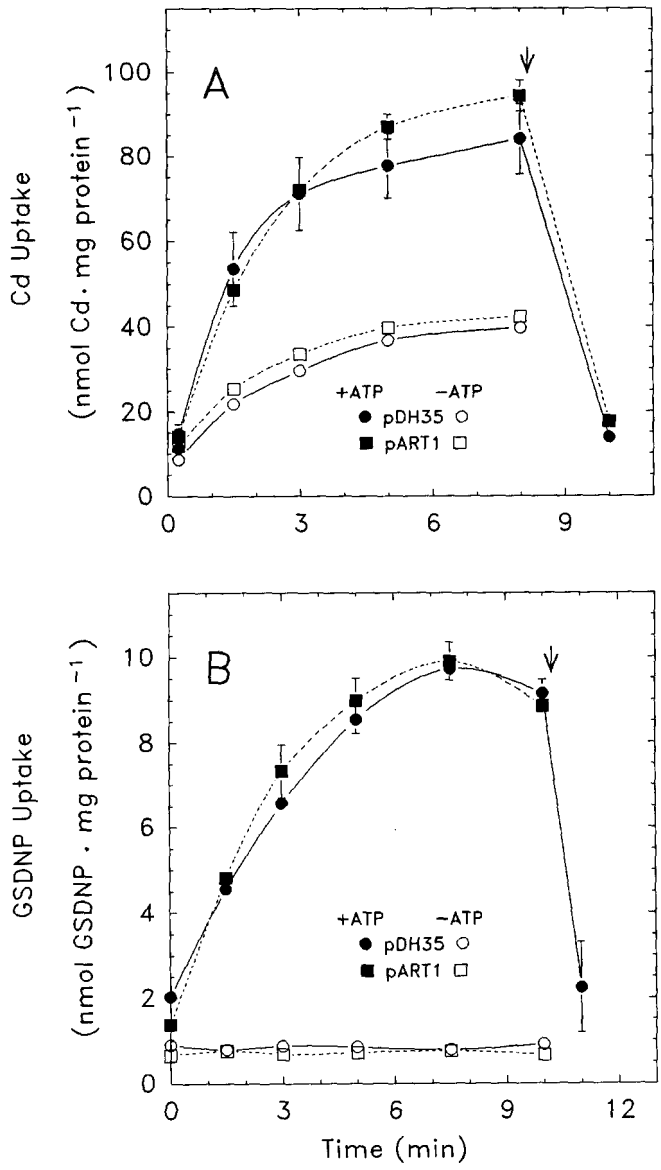

FIG. 6. **Vacuolar vesicle uptake of substrates related to PC complexes.** Vesicles derived from LK100/pDH35 (●, ○) and LK100/pART (■, □) were tested as in Fig. 2*A* in the presence (●, ■) or absence (○, □) of 3 mM ATP for uptake of the following substrates: *A*, 8 μM $^{109}CdCl_2$ (100 mCi/mmol); *B*, 50 μM [^{3}H]GSDNP. *Arrow* indicates addition of Triton X-100 to 0.02%. *Bars* represent S.E.

TABLE III
HMT1 dependent uptake of LMW [^{109}Cd]PC·Cd

LK100/pDH35 and LK100/pART vacuolar vesicles were incubated with 8 μM ^{109}Cd in the form of either $^{109}CdCl_2$ or LMW [^{109}Cd]PC·Cd. ATP dependent uptake velocity of ^{109}Cd (nmol/min/mg ± S.E.) was measured as described in Table II. Where indicated bafilomycin was present at 1 μM and Na_3VO_4 at 100 μM.

	$^{109}CdCl_2$		LMW [^{109}Cd]PC-Cd	
	Inhibitor	Bafilomycin	Bafilomycin	Bafilomycin + vanadate
LK100/pDH35	29 ± 3	<0.1[a]	6.4 ± 0.3	2.0 ± 0.4
LK100/pART	37 ± 1	<0.1[a]	0.9 ± 0.6	ND[b]

[a] Initial velocity of Cd uptake in the presence of these inhibitors was usually equal or slightly less than the binding observed in the absence of ATP.
[b] ND, indicates value not determined.

uptake of LMW [^{35}S]PC·Cd. These data are consistent with HMT1-mediated uptake of PC·Cd complexes by vacuolar vesicles. To determine if the Cd^{2+}/H^+ antiporter is capable of recognizing Cd^{2+} bound to PCs, LK100/pART vesicles were incubated with LMW [^{109}Cd]PC·Cd in the absence of bafilomycin. Uptake of the ^{109}Cd label under these conditions (7.1 ± 0.3 nmol/min/mg) was only 19% of that observed when Cd^{2+} is presented as the free metal ion (37 ± 1 nmol/min/mg).

Cysteine, GSH, and Dinitrophenyl-GSH—PCs are synthesized from GSH, and are in a sense GSH polymers. However, vacuolar membrane vesicles do not take up [^{3}H]GSH in either the reduced (GSH) or oxidized (GSSG) forms (not shown). GSH itself has affinity for heavy metals, and the *Candida glabrata* HMW PC·Cd·S^{2-} complex has been shown to contain GSH (25). However, [^{3}H]GSH was not taken up by vacuolar vesicles in the presence of Cd^{2+}. This was also true when [^{35}S]cysteine was tested as a possible substrate.

Recent reports indicate that plant vacuoles contain a transporter that recognizes various GSH conjugates (40), including GSDNP which is recognized with high affinity. HMT1 function might be a part of a more generalized xenobiotic detoxification pathway involving sequestration of GSH conjugates produced by glutathione *S*-transferases. Vacuolar membrane vesicles exhibited ATP-dependent [^{3}H]GSDNP uptake (Fig. 6*B*) that was abolished at 0 °C, or when ATP was substituted with AMP, ADP, or AMP-PNP. Unlike PC transport, however, vacuolar vesicles derived from LK100/pART1 or LK100/pDH35 exhibited no significant difference regarding [^{3}H]GSDNP uptake. Thus, fission yeast vacuoles contain a GSH-conjugate transporter that is distinct from HMT1. GSDNP transport was similar to HMT1-mediated PC uptake in that it was not inhibited by proton ionophores or bafilomycin, but was significantly reduced in the presence of 0.1 mM vanadate (not shown).

DISCUSSION

It has been established that PCs are essential for heavy metal tolerance in *S. pombe* (13), and may also play an important role in plants (12). However, PC synthesis in and of itself is not sufficient to confer heavy metal tolerance. Yeast strains harboring a mutant *hmt1* allele are Cd^{2+} sensitive, notwithstanding their ability to synthesize PCs. In this work we have shown that the native HMT1 protein is sorted to the vacuolar membrane of *S. pombe*. We also report the discovery of a novel PC transport activity in *S. pombe* vacuolar membrane vesicles. PC uptake correlates with HMT1 abundance and is inhibited by antibodies directed against this protein, indicating that HMT1 is most likely responsible for PC transport into the vacuole. PC uptake by vacuolar vesicles appears to be directly energized by ATP hydrolysis, as expected of an ABC-type transporter. It is not dependent on activity of the vacuolar proton ATPase, or sensitive to ionophores that dissipate the ΔpH or membrane potential. Similar to other ABC-type proteins, such as the cystic fibrosis transmembrane regulator (41) and P-glycoprotein (42), HMT1-mediated PC uptake is supported by ribonucleotides other than ATP and is inhibited by vanadate.

HMT1 is capable of transporting both apo-PCs (measured as uptake of [^{35}S]PC) and PC·Cd complexes (measured as uptake of LMW [^{109}Cd]PC·Cd). The HMW PC·Cd·S^{2-} complex is also taken up by vacuolar vesicles containing HMT1, albeit with lower efficiency. Some ABC-type transporters exhibit wide substrate specificity. The multiple drug resistance P-glycoprotein, for example, recognizes many structurally-unrelated drugs, and the TAP1 and TAP2 transporters mobilize peptides sharing very little, if any, amino acid sequence similarity (43). Thus, recognition of various types of PC substrates by HMT1 would not be unprecedented. However, further study is required to determine if the HMW PC·Cd·S^{2-} complex or apo-PCs represent bona fide substrates *in vivo*. The simplest model for the role of HMT1 in heavy metal tolerance involves trans-

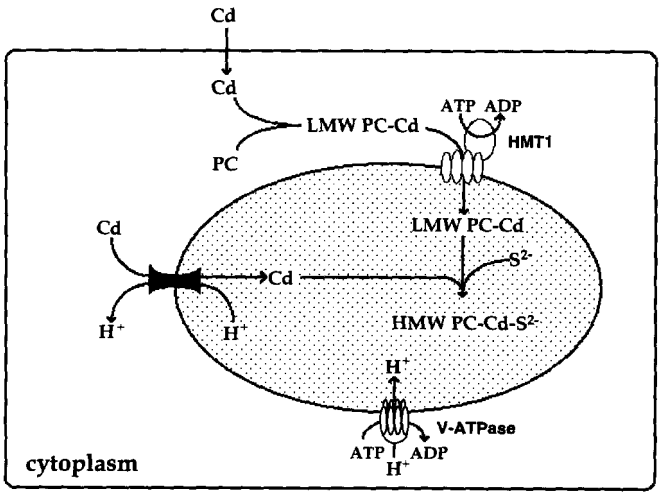

FIG. 7. **A model of PC-mediated cadmium tolerance.** Cd^{2+} ions taken up by the cell activate PC synthase (44) and induce synthesis of PCs, which chelate cytoplasmic Cd^{2+} to form the LMW PC·Cd complex. HMT1 actively transports the LMW PC·Cd complex across the vacuolar membrane and sulfide is added within the vacuole to generate the stable HMW PC·Cd·S^{2-} complex. In this model, LMW PC·Cd functions as a cytoplasmic carrier, whereas the HMW PC·Cd·S^{2-} complex is the storage form of the metal. Since the ratio of Cd^{2+} to PC is higher in the HMW complex than in the LMW complex, additional Cd^{2+} could be supplied by the Cd^{2+}/H^+ antiporter. Apo-PCs may also be transported by HMT1, while Cd^{2+} enters through the Cd^{2+}/H^+ antiporter.

port of PC-metal complexes into the vacuole as illustrated in Fig. 7. The accumulation of PCs in the vacuoles of Cd^{2+}-treated plant seedlings (45) might be explained by the presence of an HMT1-like activity in the tonoplast.

HMT1 may not be dedicated exclusively to heavy metal tolerance. Micronutrients such as copper or zinc, complexed with PCs, might be stored in the vacuole via HMT1 transport and released to the cytoplasm as required. PC·Cu and PC·Zn complexes, which can activate some apo-metalloenzymes as efficiently as free metals (46), have been shown to be a less toxic source of cofactors than free metal ions (47).

S. pombe vacuolar vesicles also exhibit ATP-dependent uptake of ^{109}Cd in the absence of PCs. However, this activity is not attributable to HMT1 as it is present at approximately the same level in a strain that hyperproduces this protein and a mutant which does not contain detectable levels of the HMT1 polypeptide. Cd^{2+} uptake appears to be mediated by a Cd^{2+}/H^+ antiporter that depends on the pH gradient generated by a vacuolar proton ATPase. A similar activity residing in the tonoplast of oat roots has been described (48). The role of the Cd^{2+} transporter in heavy metal tolerance remains unclear. Sequestration of free Cd^{2+} ions in the vacuole should ameliorate their toxicity. However, LK100 cells, which contain an active Cd^{2+} transporter, are still extremely sensitive to Cd^{2+}. Plant cells exposed to Cd^{2+} contain very little, if any, of the free metal ion (47). Intracellular Cd^{2+} appears to be found almost exclusively as PC·Cd complexes. This is in accordance with our observations which indicate that *S. pombe* extracts contain very little free Cd^{2+} (not shown). Furthermore, we find that PC bound Cd^{2+} is not an efficient substrate for the Cd^{2+} transporter. Thus, the major route for sequestration of Cd^{2+} in the vacuole may be uptake of PC·Cd complexes by HMT1. It is possible that the Cd^{2+} transporter is required when heavy metal first enters the cell and PC levels are low, or when a metal challenge exhausts the short-term capacity of the cell to synthesize stoichiometric amounts of PCs. Alternatively, Cd^{2+} may represent an adventitious substrate and the "Cd^{2+}" transporter may be involved in some aspect of metal homeostasis unrelated to Cd^{2+} tolerance. In *S. cerevisiae* and *N. crassa*, a battery of antiporters maintain vacuolar pools of Ca^{2+}, polyphosphate, and various amino acids, helping to regulate the cytoplasmic levels of these substances (reviewed in Ref. 49). A metal antiporter may serve a similar function.

To our knowledge, HMT1 represents the first reported ABC-type transporter conferring tolerance to a toxic substance by sequestration in an intracellular membrane-bound compartment. The *pfmdr1* gene product, implicated in chloroquine resistance, is localized to the digestive vacuole of *Plasmodium falciparum* (50); however, hyperproduction of this protein results in a 40–50-fold decrease in intracellular levels of the drug. *S. pombe* vacuoles also contain a glutathione conjugate transporter, which like HMT1 is insensitive to inhibitors of the vacuolar ATPase and sensitive to vanadate, suggesting by analogy that an ABC-type protein might be responsible. Recently, transport activities for glutathione conjugates (40) and bile acids (51) were found in the plant tonoplast. In both cases, it was hypothesized that ABC-type proteins were involved. Organisms are exposed to a large number of toxic compounds of both exogenous and endogenous origin. Conjugation of the toxins or their derivatives with ubiquitous intracellular metabolites such as glutathione, glucuronate, and sulfate is one way in which cells deal with this problem. In animals, a number of these conjugates are exported from the cell and then excreted from the organism. It appears that in plants and fungi, toxins "tagged" by conjugation (or chelation in the case of heavy metals) are sequestered in the vacuole, and that ABC-type transporters may play a major role in these detoxification pathways.

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